Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Research 339 (2004) 2541–2547
Carbohydrate RESEARCH

Complementary exploration of the action pattern of hyaluronate lyase from *Streptococcus agalactiae* using capillary electrophoresis, gel-permeation chromatography and viscosimetric measurements

Andrea V. Kühn,[a] Jörg-H. Ozegowski,[b] Gundela Peschel[c] and Reinhard H. H. Neubert[a,*]

[a]*Institute of Pharmaceutics and Biopharmaceutics, Martin-Luther-University Halle-Wittenberg, Wolfgang-Langenbeck-Street 4, 06120 Halle (S.), Germany*
[b]*Institute for Biochemistry 2, Friedrich-Schiller-University Jena, Am Nonnenplan 2, 07745 Jena, Germany*
[c]*Hans-Knöll-Institut für Naturstoff-Forschung e.V., Beutenbergstrasse 11, 07745 Jena, Germany*

Received 3 February 2004; accepted 16 August 2004
Available online 21 September 2004

Abstract—Hyaluronic acid (HA) was treated with hyaluronate lyase (GBS HA lyase, E.C. 4.2.2.1, from *Streptococcus agalactiae* strain 4755), and the products have been analyzed by capillary electrophoresis (CE–UV and online CE–ESIMS), gel-permeation chromatography (GPC) and viscosimetric measurements. The resulting electropherograms showed that the enzyme produced a mixture of oligosaccharides with a 4,5-unsaturated uronic acid nonreducing terminus. More exhaustive degradation of HA led to increasing amounts of di-, tetra-, hexa-, octa- and decasaccharides. Using CE, linear relationships were found between peak area of the observed oligosaccharides and reaction time. Determination of viscosity at different stages of reaction yielded an initial rapid decrease following Michaelis–Menten theory. A reaction time-dependent change in the elution position of the HA peak due to partial digestion of HA with GBS hyaluronate lyase has been observed by GPC. These results indicated that the HA lyase under investigation is an eliminase that acts in a nonprocessive endolytic manner, as at all stages of digestion a mixture of oligosaccharides of different size were found. For GBS HA lyase from *Streptococcus agalactiae* strain 3502, previously published findings reported an action pattern that involves an initial random endolytic cleavage followed by rapid exolytic and processive release of unsaturated disaccharides. Our results suggest that differences between the two enzymes from distinct *S. agalactiae* strains (GBS strains 4755 and 3502) have to be considered.

Keywords: Hyaluronic acid; Hyaluronate lyase; *Streptococcus agalactiae*; Capillary electrophoresis; GPC; Viscosity

1. Introduction

Hyaluronic acid (HA) is a high-molecular weight, naturally occurring linear polysaccharide. The repeating disaccharide units consist of *N*-acetylglucosamine (GlcNAc) and glucuronic acid (GlcA) via a β-(1→4)-linkage. HA has been assigned a variety of important physiological functions. As a component of the extracellular matrix, it maintains water balance and is important in cellular interactions. HA is known to exert a protective effect against oxidative damage of cells.[1]

Its depolymerization is catalyzed by a group of enzymes termed hyaluronidases. The enzymes are classified into three main families according to their catalytic mechanism, that is, hyaluronate 4-glycanohydrolase (testicular, lysosomal and bee venom hyaluronidases, E.C. 3.2.1.35), hyaluronate 3-glycanohydrolase (leech hyaluronidase, E.C. 3.2.1.36) and hyaluronate lyase (bacterial hyaluronidase, E.C. 4.2.2.1).[2,3] The complete sequence of the *Streptococcus agalactiae hylB* gene encoding hyaluronate lyase was first reported by Gase et al.[4]

MS characterization of saturated HA oligomers derived by digestion with testicular hyaluronidase has been presented.[5] Unsaturated oligosaccharides resulting from

* Corresponding author. Tel.: +49 345 5525001; fax: +49 345 5527292; e-mail: neubert@pharmazie.uni-halle.de

0008-6215/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2004.08.009

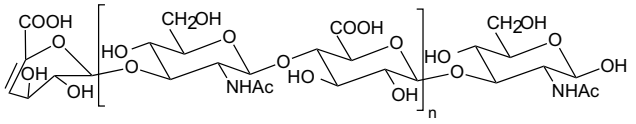

Figure 1. Structure of hyaluronic acid fragments with a 4,5-unsaturated uronic acid nonreducing terminus composed of a disaccharide repeat unit of D-glucuronic acid (GlcA) and 2-acetamido-2-deoxy-D-glucose (*N*-acetylglucosamine, GlcNAc).

enzymatic degradation with hyaluronate lyase from *S. hyalurolyticus* and *S. agalactiae* have also been analyzed.[6–8] The former HA lyases are *endo*-hexosaminidases, producing even-numbered fragments of the structure L-4dthrHex4enA-(1→3)-[D-GlcNAc-(1→4)-D-GlcA]$_n$-(1→3)-D-GlcNAc. These are accompanied by minor amounts of odd-numbered oligomers of the structure L-4dthrHex4enA-(1→3)-[D-GlcNAc-(1→4)-D-GlcA]$_n$.[7,8]

Biological functions such as functional maturation of dendritic cells during inflammation,[9] inhibition of anchorage-independent growth of tumour cells[10] and protection of granulation tissue from oxygen free radical damage[11] have been ascribed to mixtures of hyaluronic acid fragments (HAF).

For the analysis of complex mixtures of HAF from digests, separation is usually required. Owing to the charged nature of the analytes and the broad range of chain lengths, capillary electrophoresis is the method of choice. In contrast to neutral glycans,[12] HAF molecules can be separated by CE without derivatization.[13] However, UV detection does not provide specific structural information, and no standard oligomers are available that would allow a proper assignment according to the observed electrophoretic mobility. Therefore, CE–MS coupling appeared to be an alternative method.[14–16]

This study follows the lyase-catalyzed breakdown of HA by using GPC and viscosimetric assays and examines the composition of the product mixture as a function of time by CE with peak identification by online CE–ESIMS.

Although the action pattern of hyaluronan lyase from group B streptococci on HA was previously suggested to be exolytic, yielding 4,5-unsaturated disaccharides only,[17–20] our results indicate that the investigated enzyme acts as an endoglycosidase producing mixtures of terminally 4,5-unsaturated oligosaccharides (Fig. 1).

2. Experimental

2.1. Materials

Ammonium acetate, acetone and 4,5-unsaturated hyaluronic acid disaccharide (δDiHA) standard were obtained from Sigma Chemical Co. (Taufkirchen, Germany). Methanol was supplied by J. T. Baker (Deventer, The Netherlands). Sodium nitrate was from VWR International GmbH (Dresden, Germany). Pullulan standards with peak molecular masses in the range of 5.9×10^3–7.88×10^5 were from Polymer Standard Service (PSS, Mainz, Germany). Hyaluronic acid from *S. zooepidemicus* was purchased from Aqua Biochem GmbH (Dessau, Germany). Hyaluronate lyase from *S. agalactiae*, GBS strain B 4755 was produced and purified as previously described.[21]

2.2. Enzymatic depolymerization

Hyaluronic acid (250 g) was treated with 10,000 U of HA lyase from *S. agalactiae* in 50 L of 0.01 M sodium acetate–acetic acid buffer, pH 6.0. After incubation for 6 h at 25 °C, the reaction mixture was heated at 75 °C for 10 min to inactivate the enzyme, subsequently ultrafiltered and lyophilized.

2.2.1. Preparation 1. The lyophilized mixture (25 g) was suspended in 1 L of 0.01 M sodium acetate–acetic acid buffer, pH 6.0, and the mixture was stirred for 60 min at 25 °C. To the clear solution thus formed were added 4000 U HA lyase. At selected time intervals 200 mL of the reaction mixture were taken out and heated at 80 °C for 5 min to stop the enzymatic reaction. The samples were then analyzed by GPC and subsequently lyophilized.

2.2.2. Preparation 2. Digestion was carried out as described for preparation **1** using 5000 U HA lyase.

2.3. Capillary electrophoresis (CE)

CE experiments were performed according to Kühn and co-workers[15,16] using a Hewlett–Packard ^{3D}CE system (Agilent, Waldbronn, Germany) fitted with an on-column diode-array detector (190–600 nm). A kit provided by Agilent was used to modify the Hewlett–Packard ^{3D}CE system for CE–MS coupling. CE with UV detection was carried out using uncoated fused-silica capillaries, 50 μm i.d. × 360 μm o.d. × 80 cm total length (71.5 cm to the UV detector), from CS-Chromatographie Service GmbH (Langerwehe, Germany). For CE–MS analysis, polyacrylamide-coated fused-silica capillaries (50 μm i.d. × 360 μm o.d. × 90 cm total length; from CS) were used. Samples (0.5–1% HAF in CE buffer (w/v)) were injected by pressure (50 mbar, 15 s). Separations were performed at 25 °C with +30 kV at the capillary inlet. Uncoated capillaries were preconditioned for 15 min with 1 M NaOH before the first run. Prior to each subsequent run the capillaries were rinsed with 0.1 M NaOH and buffer solution for 5 min. Coated capillaries were conditioned with water and buffer each for 5 min. The

running electrolyte was 40 mM ammonium acetate buffer, pH 9.0, which was degassed by ultrasound before use.

2.4. Electrospray-ionization mass spectrometry (ESIMS)

Mass spectrometry was performed using a Finnigan LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA) via the electrospray interface. The electrospray voltage applied to the ESI needle was −4.0 kV. Spectra were collected in the negative-ion ESI mode. A coaxial sheath liquid (1% Et_3N in MeOH (v/v)/water, 80:20 (v/v)) at a flow rate of 10 μL/min was used to establish a stable spray. The sheath gas was set to 0.9 L/min, and the auxiliary gas was 6 L/min (60 and 20 arbitrary units). The heated capillary was maintained at 260 °C.

2.5. Gel-permeation chromatography (GPC)

Gel-permeation chromatography was carried out with an HPLC pump system 422S (Bio-Tek Kontron Instruments, Neufahrn, Germany) consisting of a 420 pump, a DG 980–50 degasser, an HPLC 360 autosampler and an RI-930 differential refractometer (Jasco, Tokio, Japan). The mobile phase was 0.1 M sodium nitrate at a flow rate of 1 mL/min, and the analyses were performed at 25 °C. The sample solution (0.02 mL, 2.5% (w/v)) was injected onto a HEMA Bio 2000 column (300 × 8 mm i.d.) (PSS, Mainz, Germany). Values of 0.146 (pullulan) and 0.153 (HAF) were used for the refractive index increment (dn/dc). Calibration was carried out using pullulan standards.

2.6. Viscosity

Viscosity of the sample solutions was measured using a rheometer of Couette design (Rheometrics Fluids Spectrometer RFS2, Rheometric Scientific, Piscataway, NJ, USA). The sample solutions were prepared by dissolving the lyophilized digestion products in water (2.5% (w/v)). The temperature was set to 25 °C, and shear rates in a range of 0.1–100 s^{-1} were applied. Each analysis was performed three times.

3. Results and discussion

To monitor the extent of degradation of HA by GBS hyaluronate lyase, the viscosity of HAF solutions was measured. The rate of decrease in viscosity of the substrate solution corresponds to the number of cleavages of glycosidic bonds per unit time. At the concentrations studied HAF solutions behave as Newtonian solutions. In Figure 2 the viscosity at a shear rate of 63.1006 s^{-1} is plotted as a function of reaction time. It can be observed that viscosity drops rapidly following Michaelis–Menten theory. The parameters were obtained by nonlinear regression analysis of the data (Fig. 2) according to the following equation:

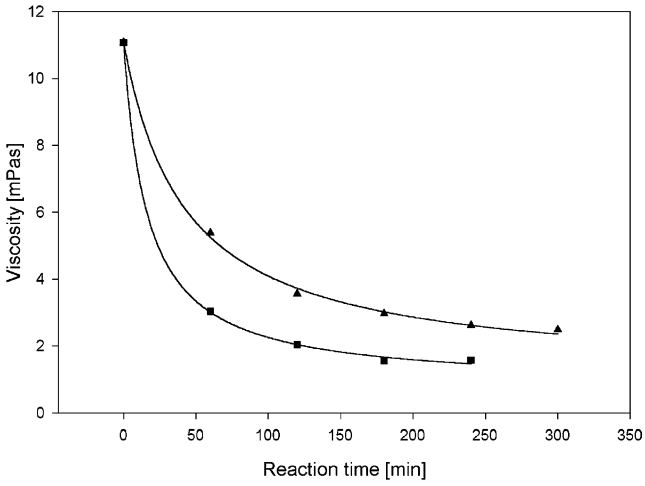

Figure 2. Relationship between reaction time and viscosity (average of three consecutive measurements) at a shear rate of 63.1006 s^{-1}. A hyperbolic decay (Eq. 1) was constructed through the datapoints. (▲) preparation **1**: $a = 9.95$ mPa s, $b = 42.54$ min, $y_0 = 1.13$ mPa s; (■) preparation **2**: $a = 10.25$ mPa s, $b = 16.37$ min, $y_0 = 0.82$ mPa s.

$$y = \frac{ab}{b + x} + y_0 \quad (1)$$

where y_0 is the viscosity of solvent (water), a is the source viscosity without y_0 and b is the time within $y = \frac{a}{2} + y_0$. From the data it is evident that the parameter b decreases about 2.5 times when the amount (U) of the enzyme used raises about 1.25 times. The rapid initial drop in viscosity confirmed the random endolytic action pattern where the enzyme selects a polysaccharide chain and cleaves the chain into oligosaccharide product and polysaccharide chain. In case of acting in an exolytic fashion, where the enzyme selects a single polysaccharide chain and removes one disaccharide unit at a time from the end of the chain, shallow concave curves (the rate of change in viscosity decreases throughout the reaction) are expected.[22]

Additionally, the samples were fractionated by GPC. This method has been widely used for the characterization of HA and its degradation products.[22–28] The chromatograms (Figs. 3 and 4) show the reaction time-dependent change in the elution position of the HA peak due to the appearance of intermediate size hyaluronan fragments during partial digestion with GBS hyaluronate lyase. Upon further action of the enzyme, an increasing peak corresponding to smaller oligosaccharides can be observed (Figs. 3 and 4). Unfortunately, a negative peak at the possible elution position of unsaturated disaccharides caused by coelution of water (sample solution) occurred.

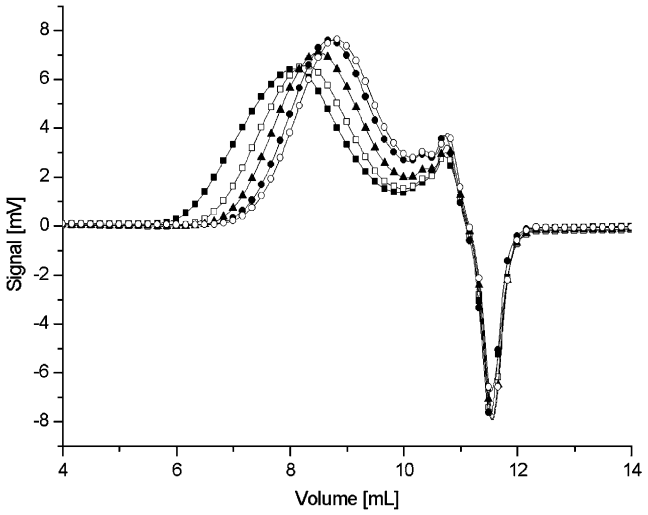

Figure 3. GPC chromatograms of preparation **1**. Reaction time: (■) 0 min, (□) 60 min, (▲) 120 min, (●) 240 min and (○) 300 min.

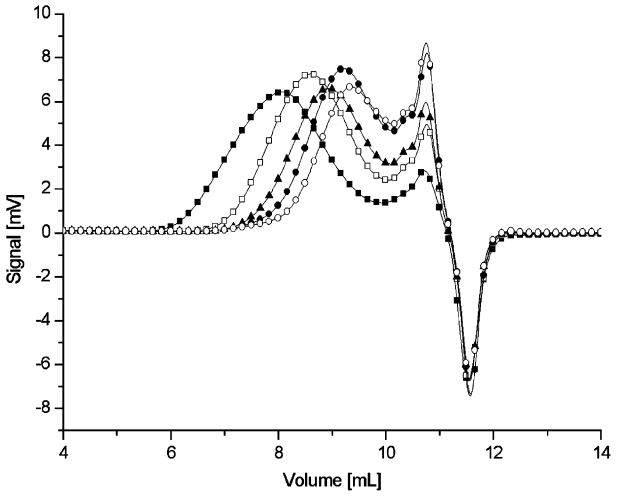

Figure 4. GPC chromatograms of preparation **2**. Reaction time: (■) 0 min, (□) 60 min, (▲) 120 min, (●) 180 min and (○) 240 min.

Degradation of HA by exoglycosidases yields only disaccharides and the excluded peak of large hyaluronic acid, but not of intermediate size products.[22,28] Since partial digestion with the previously studied GBS HA lyase from *S. agalactiae* strain 3502 yielded only unsaturated disaccharides (and the excluded peak of large HA), but none of the intermediate size HA fragments, the authors suggested that the enzyme functions as an exoglycosidase that processively moves along the chain and simultaneously releases disaccharide units from the ends of the hyaluronan chain.[17]

It is much more informative to directly monitor the formation and/or disappearance of product chains throughout the enzymatic reaction. Such detailed measurement provides substantially more information on the action pattern of the enzymes. As already pointed out above, complex mixtures of HAF from digests usually require separation. Therefore, CE has been applied for the analysis of HA digestion products, especially to study the oligosaccharides, which were insufficiently analyzed by GPC. Figure 5 shows CE–UV electropherograms of HAF at several digestion times. The electro-

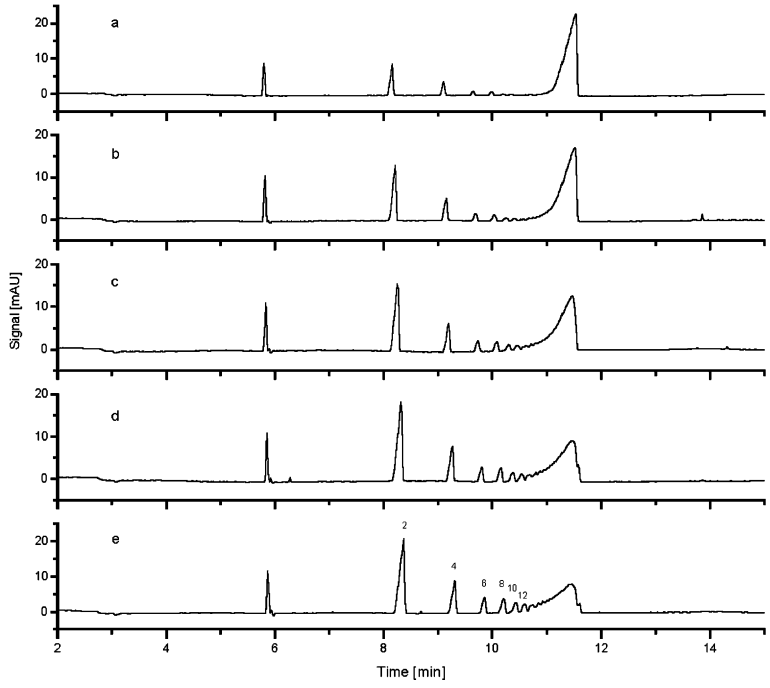

Figure 5. CE–UV electropherograms of preparation **2**, detection at 195 nm. Reaction time: (a) 0 min; (b) 60 min; (c) 120 min; (d) 180 min and (e) 240 min. Peaks labelled according to the number of monosaccharide units in the oligomer.

pherograms show a complicated mixture of oligosaccharides and a broad peak of partially digested HA. Experiments with acetone as the EOF (electroosmotic flow) marker indicated that the first peak corresponds to neutral matrix components (unidentified). δDiHA-standard comigrated with the peak marked 2. The remaining peaks were identified by online CE–ESIMS (Fig. 6). Since no suitable reference compounds are available CE–ESIMS is the method of choice. As described before,[7,8,15,16,29] multiply charged ions of HAF are generated under electrospray ionization. In case of 4,5-unsaturated HA oligosaccharides, therefore, different chain lengths are detected at same *m/z*. Whereas the migration in CE depends on the average charge of each oligomer (resulting from an equilibrium in the electrolyte solution), different charge states and adduct species (such as $[2M-H]^-$) can be subsequently formed in ESIMS and detected at the same migration time.[15,16] In addition, singly, doubly, triply and quadruply charged ions and adduct ions have been observed. As shown in Figures 5 and 6, the differences in migration time become smaller with increasing chain length of the oligomers. The peaks in the electropherograms all correspond to unsaturated HA oligosaccharides and no saturated ones have been found by CE–ESIMS. This is in agreement with an endolytic action pattern. Each cleavage of a hyaluronan chain results in the formation of two fragments of lower molecular mass, that is, one saturated fragment (intermediate size hyaluronan fragment) and one unsaturated oligosaccharide. The ESIMS technique allows one to detect these oligosaccharides up to the 16-mer.[7,15,16]

The comparison of CE–ESIMS with CE–UV electropherograms showed good similarities in the peak profiles. Therefore, the peak areas corresponding to the 2-, 4-, 6-, 8- and 10-mer in all of the CE–UV electropherograms were determined and plotted as a function of reaction time (Fig. 7). A linear relationship between peak area and reaction time was found, which is not expected in the case of an exolytic mechanism where these oligosaccharides should be rapidly degraded into unsaturated disaccharides. In addition, the ratio of δDiHA to the oligosaccharides observed is too low to assume an exolytic and processive cleavage of HA by GBS lyase.

The results obtained for the investigated enzyme acting on HA (presented above) are contrary to previous observations for HA lyase from *S. agalactiae* (strain 3502) and will be, therefore, discussed below.

The predigestion and lyophilization of hyaluronan as described in Section 2 has been essential for handling the material, especially for the viscosity measurements. Although the material has already been reduced to intermediates, this does not affect the following studies, since

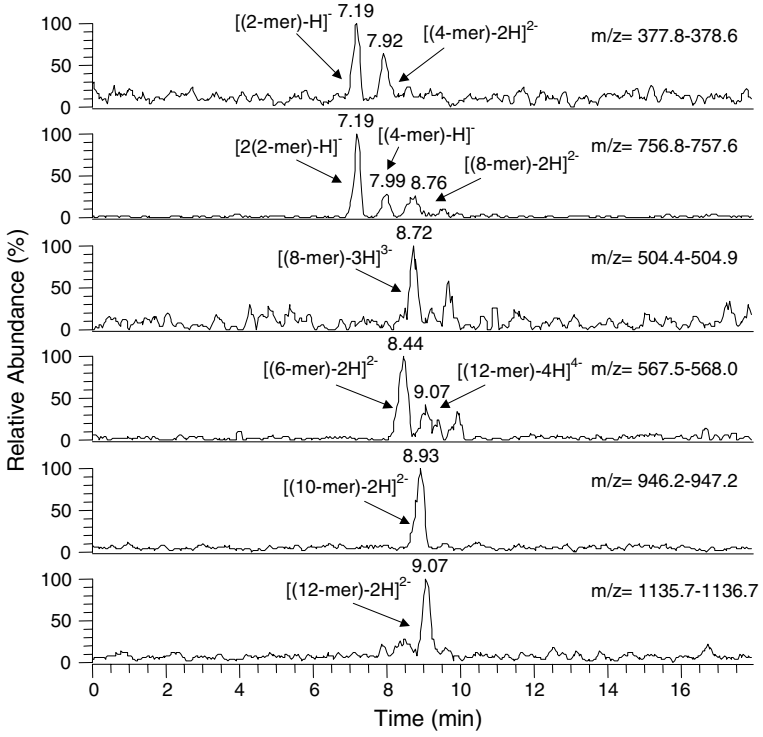

Figure 6. CE–MS of preparation **2** (reaction time 240 min). MS acquisition was started 1 min after starting the CE run. Extracted ion electropherograms of 2-mer (at approx. 7.19 min), 4-mer (at approx. 7.92–7.99 min), 6-mer (at approx. 8.44 min), 8-mer (at approx. 8.72–8.76 min), 10-mer (at approx. 8.93 min) and 12-mer (at approx. 9.07 min).

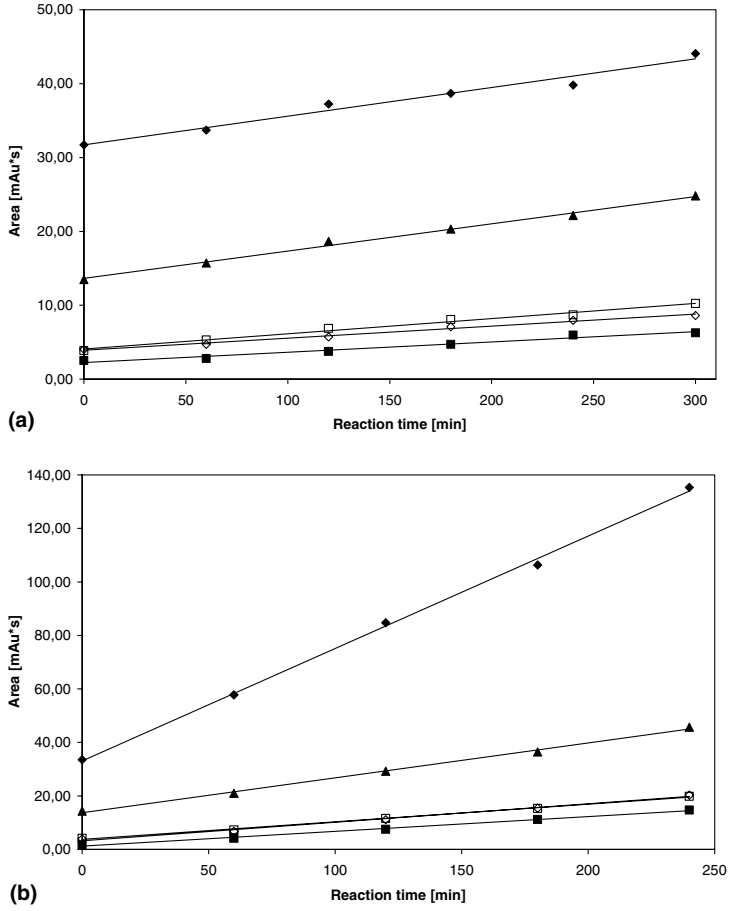

Figure 7. Linear relationships between reaction time and averages of peak areas from five consecutive CE–UV electropherograms during the degradation of HA by GBS hyaluronate lyase. (a) Preparation **1**; (b) preparation **2**: (♦) 2-mer, (▲) 4-mer, (□) 6-mer, (◊) 8-mer, (■) 10-mer.

the used enzyme is the same throughout the degradation periods. The investigated GBS HA lyase is a basic protein (isoelectric point (p*I*) at pH 8.75[21]), and maximum activity of the enzyme was found at pH 6.3[21] and 5.0.[30] Therefore, the degradation process in this study has been carried out at pH 6.0. Investigations of degradation products by GPC[28] and MALDI-TOF-MS[8] (degradation conditions: pH 7, 37 °C and pH 7, 30 °C, respectively, which were applied in order to generate conditions similar to physiological ones) also revealed mixtures of oligosaccharides and intermediates of different sizes at all stages of digestion. In the investigated partial digests of these studies, the tetra-, hexa- and octasaccharide tend to accumulate rather than the unsaturated disaccharide.[8,15]

The mentioned conditions, as well as the addition of calcium or magnesium ions, influence the velocity of degradation but not the action mechanism of the enzyme. A proteolytic cleavage of the mature protein (118 kDa) to smaller fragments (110 and 94 kDa) has been reported. However, this does not result in a loss of processivity since all three polypeptides were enzymatically active.[4]

The investigated enzyme is known to act in extremely fast fashion,[30] which is in agreement with the catalytic mechanism found in this work.

Although the enzyme investigated in the present study showed extensive sequence identity to the previously studied homologous enzyme from GBS strain 3502,[4,31] differences in the enzymes (due to mode of isolation or source) must be suspected.

Further investigations regarding the exact reason for the difference between the two HA lyases from serologically and epidemiologically distinct *S. agalactiae* strains have to be carried out.

4. Conclusions

HA was depolymerized with hyaluronan lyase from group B streptococci, and the degradation products were analyzed by CE, online CE–ESIMS, GPC and viscosity measurements. From the data it is evident that the molecular weight of native HA was strongly reduced during the digestion period. Increasing the enzyme units used for the digestions resulted in more rapid degrada-

tion of HA and led to increasing amounts of unsaturated oligo- and disaccharides. The application of both methods, GPC and CE, offered the ability to obtain complementary information about the products derived by the enzymatic degradation of HA. Identification of peaks by online CE–ESIMS permits a definite assignment according to the observed electrophoretic mobility, which would not be possible using CE–UV. The results of this study confirmed the random endolytic action pattern of the enzyme used, which is in agreement with previous results derived by gel permeation chromatography[28] and MALDI-TOF-MS.[8]

With respect to the above-mentioned biological functions, it has to be noted, particularly for the use of hyaluronidases in pharmaceutical preparations, that hyaluronan degradation by the examined enzyme yields a mixture of oligosaccharides and HAF of different sizes.

Acknowledgements

The authors wish to thank Mrs. M. Woigk and Mrs. S. Todte for excellent technical assistance. Furthermore, the financial support of the Land Sachsen-Anhalt for the stipend of A. V. Kühn is gratefully acknowledged.

References

1. Presti, D.; Scott, J. E. *Cell Biochem. Funct.* **1994**, *12*, 281–288.
2. Linhardt, R. J.; Galliher, P. M.; Cooney, C. L. *Appl. Biochem. Biotech.* **1986**, *12*, 135–176.
3. Prehm, P. Hyaluronan. In *Biopolymers, Polysaccharides 1, Polysaccharides from Prokaryotes*; Vandamme, E. J., De Baets, S., Steinbüchel, A., Eds.; Wiley-VCH: Weinheim, 2002; Vol. 5, pp 379–406.
4. Gase, K.; Ozegowski, J.; Malke, H. *Biochim. Biophys. Acta* **1998**, *1398*, 86–98.
5. Mahoney, D. J.; Aplin, R. T.; Calabro, A.; Hascall, V. C.; Day, A. J. *Glycobiology* **2001**, *11*, 1025–1033.
6. Park, Y.; Cho, S.; Linhardt, R. J. *Biochim. Biophys. Acta* **1997**, *1337*, 217–226.
7. Price, K. N.; Tuinman, A.; Baker, D. C.; Chisena, C.; Cysyk, R. L. *Carbohydr. Res.* **1997**, *303*, 303–311.
8. Kühn, A. V.; Raith, K.; Sauerland, V.; Neubert, R. H. H. *J. Pharm. Biomed. Anal.* **2003**, *30*, 1531–1537.
9. Termeer, C. C.; Hennies, J.; Voith, U.; Ahrens, T.; Weiss, J. M.; Prehm, P.; Simon, J. C. *J. Immunol.* **2000**, *165*, 1863–1870.
10. Ghatak, S.; Misra, S.; Toole, B. P. *J. Biol. Chem.* **2002**, *277*, 38013–38020.
11. Trabucchi, E.; Pallotta, S.; Morini, M.; Corsi, F.; Franceschini, R.; Casiraghi, A.; Pravettoni, A.; Foschi, D.; Minghetti, P. *Int. J. Tissue React.* **2002**, *24*, 65–71.
12. Gennaro, L. A.; Delaney, J.; Vouros, P.; Harvey, D. J.; Domon, B. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 192–200.
13. Grimshaw, J.; Kane, Á.; Trocha-Grimshaw, J.; Douglas, A.; Chakravarthy, U.; Archer, D. *Electrophoresis* **1994**, *15*, 936–940.
14. Raith, K. Hyphenation of ACE with Mass Spectrometry. In *Drugs and the Pharmaceutical Sciences, Affinity Capillary Electrophoresis in Pharmaceutics and Biopharmaceutics*; Neubert, R. H. H., Rüttinger, H. H., Eds.; Marcel Dekker: New York, 2003; Vol. 128, pp 329–349.
15. Kühn, A. V.; Rüttinger, H. H.; Neubert, R. H. H.; Raith, K. *Rapid Commun. Mass Spectrom.* **2003**, *17*, 576–582.
16. Kühn, A. V.; Ozegowski, J. H.; Neubert, R. H. H. *Rapid Commun. Mass Spectrom.* **2004**, *18*, 733–734.
17. Pritchard, D. G.; Lin, B.; Willingham, T.; Baker, J. R. *Arch. Biochem. Biophys.* **1994**, *315*, 431–437.
18. Baker, J. R.; Pritchard, D. G. *Biochem. J.* **2000**, *348*, 465–471.
19. Pritchard, D. G.; Trent, J. O.; Zhang, P.; Egan, M. L.; Baker, J. R. *Proteins* **2000**, *40*, 126–134.
20. Li, S.; Jedrzejas, M. J. *J. Biol. Chem.* **2001**, *276*, 41407–41416.
21. Ozegowski, J. H.; Günther, E.; Reichardt, W. *Zbl. Bakt.* **1994**, *280*, 497–506.
22. Jandik, K. A.; Gu, K.; Linhardt, R. J. *Glycobiology* **1994**, *4*, 289–296.
23. Vercruysse, K. P.; Lauwers, A. R.; Demeester, J. M. *J. Chromatogr. B* **1994**, *656*, 179–190.
24. Al-Assaf, S.; Williams, P. A.; Phillips, G. O. Molecular Characterisation of Hyaluronan and Hylan using GPC-MALLS and Asymmetrical Flow FFF-MALLS. In *Hyaluronan (Proceedings of the International Cellucon Conference 2000)*; Kennedy, J. F., Phillips, G. O., Williams, P. A., Eds.; Woodhead: Cambridge, UK, 2002; Vol. 1, pp 55–65.
25. Chabreček, P.; Šoltés, L.; Orviský, E. *J. Appl. Polym. Sci. Appl. Polym. Symp.* **1991**, *48*, 233–241.
26. Orviský, E.; Šoltés, L.; Al-Assaf, S. *Chromatographia* **1994**, *39*, 366–368.
27. Šoltés, L.; Mendichi, R.; Lath, D.; Mach, M.; Bakoš, D. *Biomed. Chromatogr.* **2002**, *16*, 459–462.
28. Rodig, H.; Ozegowski, J. H.; Peschel, G.; Müller, P. J. *Zbl. Bakt.* **1999**, *289*, 835–843.
29. Prebyl, B. S.; Kaczmarek, C.; Tuinman, A. A.; Baker, D. C. *Carbohydr. Res.* **2003**, *338*, 1381–1387.
30. Oettl, M.; Hoechstetter, J.; Asen, I.; Bernhardt, G.; Buschauer, A. *Eur. J. Pharm. Sci.* **2003**, *18*, 267–277.
31. Lin, B.; Hollingshead, S. K.; Coligan, J. E.; Egan, M. L.; Baker, J. R.; Pritchard, D. G. *J. Biol. Chem.* **1994**, *269*, 30113–30116.